

Isolation from Animal Tissue and Genetic Transformation of *Coxiella burnetii* Are Facilitated by an Improved Axenic Growth Medium[▽]

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We recently described acidified citrate cysteine medium (ACCM), which supports host cell-free (axenic) growth of *Coxiella burnetii*. After 6 days of incubation, greater than 3 logs of growth was achieved with the avirulent Nine Mile phase II (NMII) strain. Here, we describe modified ACCM and culture conditions that support improved growth of *C. burnetii* and their use in genetic transformation and pathogen isolation from tissue samples. ACCM was modified by replacing fetal bovine serum with methyl- β -cyclodextrin to generate ACCM-2. Cultivation of NMII in ACCM-2 with moderate shaking and in 2.5% oxygen yielded 4 to 5 logs of growth over 7 days. Similar growth was achieved with the virulent Nine Mile phase I and G isolates of *C. burnetii*. Colonies that developed after 6 days of growth in ACCM-2 agarose were approximately 0.5 mm in diameter, roughly 5-fold larger than those formed in ACCM agarose. By electron microscopy, colonies consisted primarily of the *C. burnetii* small cell variant morphological form. NMII was successfully cultured in ACCM-2 when medium was inoculated with as little as 10 genome equivalents contained in tissue homogenates from infected SCID mice. A completely axenic *C. burnetii* genetic transformation system was developed using ACCM-2 that allowed isolation of transformants in about 2 1/2 weeks. Transformation experiments demonstrated clonal populations in colonies and a transformation frequency of approximately 5×10^{-5} . Cultivation in ACCM-2 will accelerate development of *C. burnetii* genetic tools and provide a sensitive means of primary isolation of the pathogen from Q fever patients.

Coxiella burnetii is a wide-ranging zoonotic pathogen that causes a debilitating influenza-like illness in humans called Q fever (20). Following infection of a eukaryotic host cell, this intracellular bacterium replicates exclusively within a phagolysosome-like parasitophorous vacuole (PV) (16). *C. burnetii* directs development of the PV (15, 24) and manipulates other host cell functions such as apoptotic signaling (18, 29). The mechanisms by which *C. burnetii* modifies the host cell and causes disease are largely unknown. Indeed, lipopolysaccharide is the only defined virulence factor of *C. burnetii* with the full-length molecule of phase I bacteria required for full virulence (21).

The obligate intracellular nature of *C. burnetii* has severely impeded development of genetic tools for virulence factor discovery. Beare et al. (3) recently genetically transformed *C. burnetii* to chloramphenicol resistance and mCherry red fluorescent protein expression using the *mariner*-based *HimarI* transposon. However, a significant limitation of the procedure is the 8 to 12 weeks required for expansion and clonal isolation of individual transposon mutants using cell culture-based prop-

agation of *C. burnetii*. Moreover, the micromanipulation method used for harvesting clonal *C. burnetii* from an individual PV is technically challenging.

The recent description of a method for axenic (host cell-free) propagation of *C. burnetii* is a significant technological advance that will facilitate biochemical and genetic studies of the pathogen (22). A growth medium called acidified citrate cysteine medium (ACCM) was developed that supports roughly 3 logs (\log_{10}) of growth of *C. burnetii* over 6 days in a microaerobic environment. Minute (approximately 0.1 mm in diameter) colonies also develop over the same time frame in semisolid ACCM agarose. In the current study, we describe a modified version of ACCM called ACCM-2 that supports improved growth of *C. burnetii* in liquid medium and as colonies in agarose plates. Colonies contained clonal populations, thereby facilitating development of a fully axenic system for *C. burnetii* genetic transformation that substantially reduces the time of transformant isolation. ACCM-2 cultures were also successfully started with infected animal tissue homogenates containing small numbers of *C. burnetii*, thereby providing a potential means of obtaining clinical isolates from Q fever patients.

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MATERIALS AND METHODS

***C. burnetii* isolates and host cell-based propagation.** *C. burnetii* Nine Mile phase II, clone 4 (NMII; RSA439), and low-passage-number stocks of Nine Mile phase I (NMI; RSA493) and G (Q212) isolates were used in this study. Host

TABLE 1. Primers used in this study

Primer	Sequence (5'-3')
CBU0316-F	GCAAAACAAGAGCAAAATATTGTTTACC
CBU0317-R	TTGATCGTTCCAGAGACAG
MCforpJB2581-F	ACAGGAAACAGAATTCAGGAGAAATTACTATGGTCTCTAAGGGCGAGGAAGACAAC
MCforpJB2581-R	CGACTCTAGAGGATCCTTATTGTACAGCTCATCCATGCC
CAT-P1169F	TCATGAAGGAGGGCCCTTATGGAGAAAAAATCACTGGATATACC
CAT-pJB2581-HindIIIrecR	GCAGGCATGCAAGCTTTTACGCCCGCCCTGCCACTC
Kan-P1169F	TCATGAAGGAGGGCCCTTATGATTGAACAAGATGGATTGC
Kan-pJB2581-HindIIIrecR	GCAGGCATGCAAGCTTTTCAAGAAGAACTCGTCAAGAAGGCGATAG
P1169-pJB2581-Ab-HindIIIrecF	CAAAACAGCCAAGCTTATGGCTTCGTTTCGACGCG
P1169-R	AAGGGCCCTCCTTCATGAAGGATTAATGTCATTATTTATTTATGGGG

cell-based growth of *C. burnetii* was accomplished using African green monkey kidney (Vero) fibroblasts (CCL-81; American Type Culture Collection) grown in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 2% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. Bacteria were purified from host cells using differential centrifugation as previously described (10) and stored at -80°C.

Axenic cultivation. A modified form of ACCM (22) called ACCM-2, in which 1% FBS is replaced with 1 mg/ml methyl- β -cyclodextrin (M β -CD), was developed. *C. burnetii* was cultivated in T-75 cell culture flasks or 0.2- μ m-pore-size-filter-capped 125-ml Erlenmeyer flasks containing 20 ml of medium, T-25 flasks containing 5 ml of medium, or 6-well tissue culture plates containing 3 ml of medium per well. Cultures were incubated at 37°C in a 2.5% O₂ and 5% CO₂ environment using a CO-170 incubator (New Brunswick Scientific, NJ). Oxygen was displaced by nitrogen gas. Where indicated, Erlenmeyer flasks were shaken at 75 rpm. *C. burnetii* replication was measured by optical density at 600 nm (OD₆₀₀) or quantitative PCR (Q-PCR) of genome equivalents (GE) using a *dotA* probe as previously described (22).

C. burnetii colonies were established using a modified soft agarose overlay method in which bacteria are grown in the top medium layer. A 0.25% ACCM-2 agarose base in 100- by 20-mm petri dishes was created by mixing 10 ml of filter-sterilized 2 \times ACCM-2 with 10 ml of 0.5% (wt/vol in water) melted Ultra-Pure agarose (Invitrogen). The bacterial inoculum was mixed with 2.5 ml of 0.1% melted ACCM-2 agarose equilibrated to 37°C, which was prepared by mixing together 1.25 ml of 2 \times ACCM-2, 0.75 ml of water, and 0.5 ml of 0.5% melted agarose. This solution was poured on top of the solidified ACCM-2 agarose base. Plates were refrigerated (4°C) for 30 min to aid solidification of the top agarose and then placed in a laminar airflow biosafety cabinet with lids ajar for 20 min to remove condensation. Plates were incubated for 6 days as described above for ACCM-2 to allow colony development.

Electron microscopy of *C. burnetii* propagated as colonies. NMII grown as colonies was obtained from ACCM-2 agarose plates incubated for 6 days. The top agarose was removed using a cell scraper, and then the agarose base was rinsed with 1 ml phosphate-buffered saline (PBS; 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 150 mM NaCl, pH 7.8). The agarose and rinse samples were combined in 2-ml microcentrifuge tubes, and then samples were disrupted by repeated pipetting to release bacteria embedded in agarose. Samples were centrifuged at 2,000 \times g for 5 min to pellet the agarose, and then supernatants were transferred to clean 1.5-ml microcentrifuge tubes that were centrifuged at 20,000 \times g for 15 min to pellet *C. burnetii*. Bacteria were fixed for 2 h in 2.5% glutaraldehyde (vol/vol) with 0.05 M sucrose in 0.1 M sodium cacodylate buffer. Subsequent postfixation steps were performed with microwave irradiation in cycles of 2 min on, 2 min off, 2 min on at 250 W under vacuum (Pelco 3451; Ted Pella, Redding, CA). Samples were then incubated in 0.5% osmium tetroxide-0.8% potassium ferricyanide in 0.1 M sodium cacodylate, followed by 1% tannic acid in distilled water and finally 1% uranyl acetate in distilled water. Samples were dehydrated in a graded ethanol series for 1 min under vacuum and then infiltrated with Spurr's resin by incubation in graded Spurr's solutions (1:3, 1:1, 3:1 [Spurr's:ethanol]) using a microwave processor at 5-min cycles before embedding in Spurr's resin. Sections (80 nm) were cut using a Leica UC6 ultramicrotome (Leica Microsystems, Vienna, Austria) and stained with 1% uranyl acetate and Reynolds lead citrate. Samples were examined with a Hitachi H-7500 electron microscope (Hitachi, Pleasanton, CA), and images were captured with a Hamamatsu XR-100 digital camera system (AMT, Danvers, MA).

Establishment of antibiotic MICs. The MICs of chloramphenicol and kanamycin required to inhibit NMII growth in ACCM-2 were determined by incubating organisms in medium containing 0.375, 0.75, 1.5, or 3 μ g/ml of chloramphenicol or 125, 250, 375, or 500 μ g/ml of kanamycin. Chloramphen-

icol and kanamycin were dissolved in ethanol and water, respectively. Solvents had no effect on *C. burnetii* growth when tested at the highest concentrations used in the assay. Growth in liquid ACCM-2 was measured by determining the OD₆₀₀s of cultures grown in 3 ml of medium in a 6-well tissue culture plate. MICs were also established for NMII growth in ACCM-2 agarose using a CFU assay.

Colony genotyping. ACCM-2 agarose was plated with equal numbers of wild-type NMII and an NMII transformant termed NMII-MC1 that contains a *HimarI* transposon insertion between CBU0316 and CBU0317 (3, 4). Fourteen random colonies were picked from plates after 6 days of growth. A Zeiss Stemi 2000 stereo microscope was used to help visualize colonies averaging 0.5 mm in diameter. Colony locations were marked, and then individual colonies were extracted from the agarose overlay using a P-1000 pipette tip. To facilitate removal of colonies, the pipette tip opening was enlarged to approximately 5 mm in diameter by cutting the tip end. These modified tips were autoclaved prior to use. Each agarose plug was added to 1.0 ml of ACCM-2 in a sterile 1.5-ml microcentrifuge tube, and organisms were released from the agarose by repeated pipetting. The mixture was then added to 2 ml of sterile ACCM-2 (3 ml total) in 6-well plates that were incubated for 6 days to expand colony populations. Cultures were genotyped for the presence of NMII or NMII-MC1 by using PCR. Two microliters of culture medium was added directly to a PCR mix that contained primers specific to CBU0316 (CBU0316-F, forward primer) and CBU0317 (CBU0317-R, reverse primer) (Table 1). PCRs were conducted using AccuPrime Taq DNA polymerase (Invitrogen), and reaction products were analyzed by gel electrophoresis using a 0.8% agarose gel. Products of 1.6 and 4.8 kb are expected from amplification of NMII and NMII-MC1 genomic DNA, respectively.

Transformation of NMII. The plasmids pJB-CAT and pJB-KAN (Fig. 1), conferring resistance to chloramphenicol and kanamycin, respectively, were used to transform NMII, and were constructed using pJB2581 as the backbone (2). The *cyaA* gene of pJB2581 was first replaced with the mCherry gene, which was amplified by PCR from p1898-Tn (3) using primers MCforpJB2581-F and MCforpJB2581-R (Table 1), and cloned into plasmid digested with EcoRI and BamHI. The chloramphenicol acetyltransferase (CAT) gene was amplified from pJB2581 using the primers CAT-P1169F and CAT-pJB2581-HindIIIrecR, and the kanamycin resistance gene (KAN) was amplified from pCR2.1-topo using the primers KAN-P1169F and KAN-pJB2581-HindIIIrecR. The CBU1169 promoter

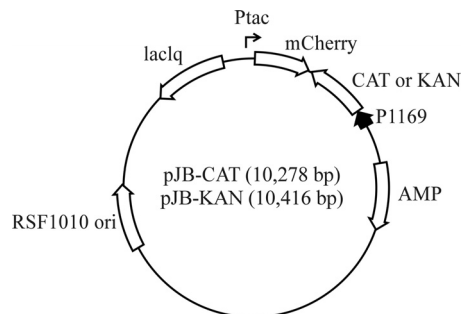


FIG. 1. Composite map of pJB-CAT and pJB-KAN. Plasmids contain a RSF1010 origin of replication and a chloramphenicol or kanamycin resistance gene under the control of the *C. burnetii* CBU1169 promoter.

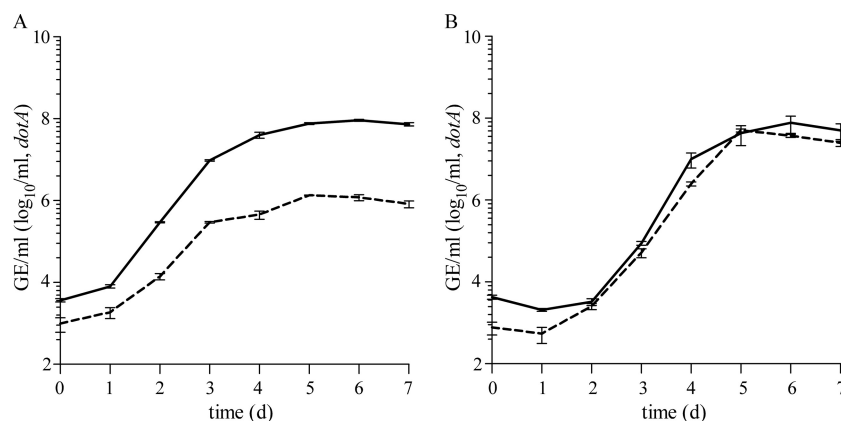


FIG. 2. Cultivation of *C. burnetii* in ACCM-2. (A) Growth of NMII in ACCM-2 with shaking (solid line) and ACCM without shaking (dotted line). (B) Growth of the virulent *C. burnetii* NMI (dotted line) and G (solid line) isolates in ACCM-2 with shaking. Growth was measured by enumerating GE, and results are from three separate cultures \pm the standard error of the mean.

(P1169) was amplified from *C. burnetii* genomic DNA using the primers P1169-pJB2581-Ab-HindIIIrecF and P1169-R. The CAT and KAN genes were placed downstream from P1169 to create P1169-CAT and P1169-KAN cassettes using overlapping PCR and the primers P1169-pJB2581-Ab-HindIIIrecF and CAT-pJB2581-HindIIIrecR or KAN-pJB2581-HindIIIrecR, respectively. P1169-CAT and P1169-KAN cassettes were cloned into HindIII-digested pJB2581::mCherry using the In-fusion kit (BD Clontech, Mountain View, CA) to create pJB-CAT and pJB-KAN, respectively. PCR was conducted with AccuPrime Pfx polymerase (Invitrogen) and primers from Integrated DNA Technologies (Coralville, IA).

Transformation experiments used NMII cultured for 6 days in ACCM-2. Cultures were grown with shaking in Erlenmeyer flasks. Bacteria were pelleted by centrifugation for 15 min at $16,000 \times g$, washed in 10% glycerol, pelleted again, and then suspended in 10% glycerol at a cell density of approximately 4×10^9 GE/ml. Organisms were kept on ice until electroporation. Fifty microliters of *C. burnetii* suspension (2×10^8 GE) was mixed with 10 μ g of pJB-CAT or pJB-KAN and placed in a 0.1-cm electroporation cuvette (precooled on ice). Cells were electroporated at 18 kV, 500 Ω , and 25 μ F for 7 to 13 ms, which results in approximately 50% killing (unpublished observations). Immediately following electroporation, 950 μ l of RPMI cell culture medium was added to the cuvette and the sample was mixed, and then 200 μ l was added to 3 ml of ACCM-2 in a 6-well plate. Cultures were incubated overnight, and then kanamycin (final concentration of 375 μ g/ml) or chloramphenicol (final concentration of 3 μ g/ml) was added to the medium for transformant selection. *C. burnetii* transformants were cultured for a total of 4 days; then, 100 μ l of the culture was plated on antibiotic-containing ACCM-2 agarose.

Genes conferring resistance to chloramphenicol, kanamycin, or ampicillin are approved for *C. burnetii* genetic transformation by the Rocky Mountain Laboratories Institutional Biosafety Committee and the Centers for Disease Control and Prevention Select Agents Program.

SCID mice infections. Six-week-old SCID mice (NOD.CB17-Prkdc^{scid}/J) were purchased from Jackson Laboratory. Three mice per group were challenged intratracheally or intraperitoneally with 10^6 GE of ACCM-2-grown NMII. At 7 days postinfection, lungs, liver, and spleen were aseptically harvested and stored at -80°C . At the time of processing, whole organs were disrupted by gentle mechanical homogenization in 5 ml of ACCM-2. To quantify bacterial load by Q-PCR, 50 μ l of tissue homogenate was processed using the High-Pure PCR template kit (Roche) and NMII GE were determined using primers specific for the *C. burnetii* *com1* gene as previously described (6). Culture positivity was determined by inoculating 5 ml of ACCM-2 in T-25 flasks with 50 μ l of 100-fold-diluted tissue homogenates containing known amounts of NMII GE. After a 7-day incubation, 1 ml of each ACCM-2 culture was centrifuged at $15,000 \times g$ for 20 min to pellet NMII. The bacterial pellet was suspended in 100 μ l of sucrose phosphate buffer (PBS containing 250 mM sucrose), and 25 μ l was processed for Q-PCR as described above. Animal experiments were performed according to protocol number AUP2009-20 approved by the University Animal Care Committee of Texas A&M University.

RESULTS

NMII growth is improved in ACCM-2. ACCM contains FBS and supports roughly 3 logs of NMII growth over a 6-day incubation without shaking (22). We found that by replacing FBS with 1.0 mg/ml M β -CD to generate ACCM-2, and by slowly shaking the cultures (75 rpm), growth yield of NMII was improved. NMII cultures were initiated with approximately 10^3 organisms/ml, and the OD₆₀₀ and GE were measured over a 7-day incubation. Cultures in ACCM without shaking and in ACCM-2 with shaking showed OD₆₀₀ increases from 0 to 0.160 ± 0.004 and 0.587 ± 0.003 , respectively. Q-PCR showed GE increases of 2.9 ± 0.075 and 4.4 ± 0.042 logs in ACCM without shaking and in ACCM-2 with shaking, respectively (Fig. 2A). The log phase (1 to 3 days postinoculation) generation time of NMII in ACCM-2 was 4.7 h, 4.4 h less than that observed in ACCM (22). Shaking of ACCM moderately improved NMII growth but not to the level of growth in shaken ACCM-2 (data not shown).

ACCM-2 supports growth of virulent *C. burnetii*. The avirulent NMII isolate was used to develop ACCM (22) and ACCM-2 culture methods. To confirm that ACCM-2 supports growth of virulent *C. burnetii* isolates, media were inoculated with the NMI and G isolates (5) and their growth was assessed. In ACCM-2, NMI and G exhibited growth yield equivalent to that observed for NMII with 4.5 ± 0.094 and 4.1 ± 0.378 logs of growth over 7 days, respectively (Fig. 2B).

Colony growth is improved in ACCM-2 agarose. NMII forms minute (approximately 0.1-mm-diameter) colonies in ACCM agarose (22). To test whether improved growth of NMII in ACCM-2 correlated with larger colonies in ACCM-2 agarose, NMII was plated and colony size was assessed after a 6-day incubation. Colonies in ACCM-2 agarose were 5-fold larger (approximately 0.5 mm in diameter) than those in ACCM agarose (Fig. 3A and B). Colonies in ACCM-2 agarose were primarily comprised of the small cell variant (SCV) form of *C. burnetii* as assessed by size and characteristic condensed chromatin (9) (Fig. 3C). Virulent NMI and G strains formed colonies in ACCM-2 agarose similar in size to those formed by NMII (data not shown).

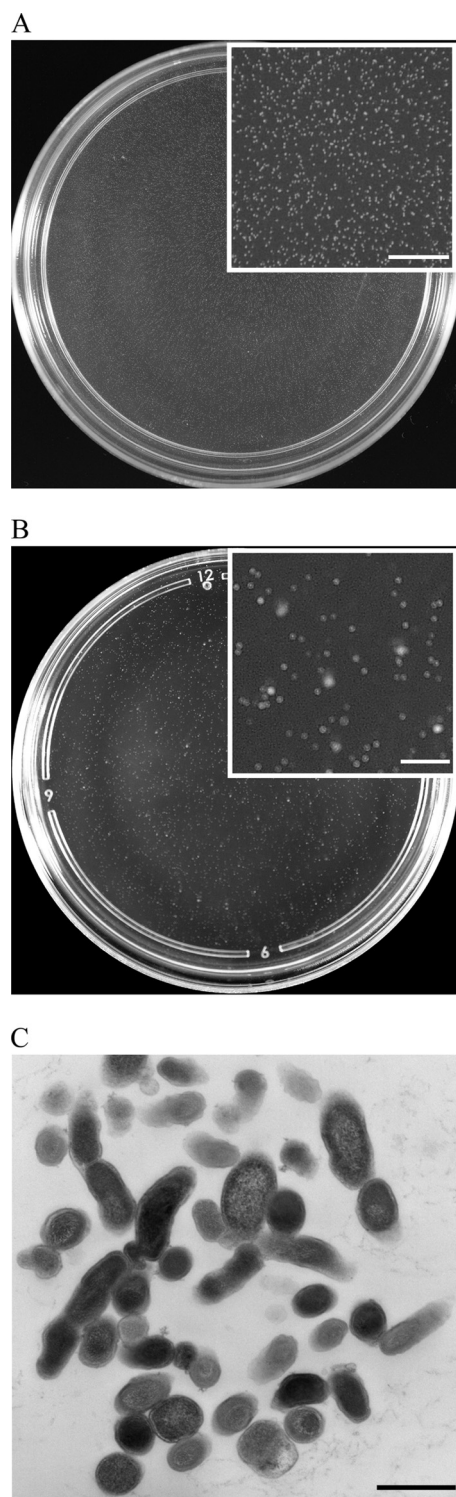


FIG. 3. Colony formation by *C. burnetii* in ACCM and ACCM-2 agarose. (A) Representative ACCM plate showing NMII colonies that average approximately 0.1 mm in diameter. Scale bar, 10 mm. (B) Representative ACCM-2 plate showing NMII colonies that average approximately 0.5 mm in diameter. Scale bar, 10 mm. (C) Electron microscopy of colony-grown *C. burnetii* showing primarily the SCV morphological form. Scale bar, 0.5 μ m.

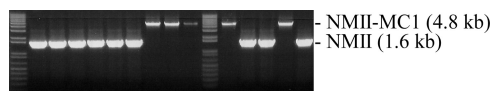


FIG. 4. NMII colonies in ACCM-2 agarose are clonal. Equal numbers of NMII and NMII-MC1, which contains a *HimarI* transposon insertion between CBU0316 and CBU0317, were plated in ACCM-2 agarose. Fourteen random colonies were picked after 6 days of growth and genotyped as described in Materials and Methods. Each colony contained either NMII (1.6-kb band) or NMII-MC1 (4.8-kb band) but not both, showing the clonality of *C. burnetii* colonies in ACCM-2 agarose.

***C. burnetii* colonies in ACCM-2 agarose are clonal.** The plating efficiency of NMII in ACCM-2 agarose was 1 to 2 GE per colony formed, suggesting that colonies develop from single organisms. To confirm clonality, ACCM-2 agarose was plated with equal numbers of wild-type NMII and a NMII transformant termed NMII-MC1 that contains a *HimarI* transposon insertion between CBU0316 and CBU0317 conferring mCherry expression and chloramphenicol resistance (3, 4). NMII-MC1 and NMII have been previously shown to grow at equal rates (4). Fourteen random colonies were picked from plates after 6 days of growth, expanded in ACCM-2, and then genotyped by PCR. Each colony contained either NMII or NMII-MC1 but not both (Fig. 4), illustrating the clonality of *C. burnetii* colonies in ACCM-2 agarose.

Culture in ACCM-2 accelerates *C. burnetii* genetic transformation. The host cell-based *C. burnetii* genetic transformation protocol described by Beare et al. (3) requires 8 to 12 weeks for clonal isolation of transformants. Therefore, we investigated whether ACCM-2 culture methods could accelerate the procedure. The first step was to determine the MICs of chloramphenicol and kanamycin in ACCM-2. NMII was cultured under different antibiotic concentrations, and growth was measured by OD₆₀₀ in ACCM-2, or colony development in ACCM-2 agarose, after a 6-day incubation. The MICs of chloramphenicol and kanamycin in ACCM-2 were 1.5 μ g/ml and 375 μ g/ml, respectively. The MICs of chloramphenicol and kanamycin in ACCM-2 agarose were 3.0 μ g/ml and 375 μ g/ml, respectively.

We next optimized a procedure for isolating *C. burnetii* transformants and established a transformation frequency. The plasmids pJB-CAT and pJB-KAN, conferring chloramphenicol and kanamycin resistance, respectively, were constructed for these experiments (see Fig. 1 and Materials and Methods). These plasmids contain an RSF1010 origin of replication that can support autonomous replication of heterologous plasmid DNA in *C. burnetii* (7). Following electroporation, optimal recovery of antibiotic-resistant colonies in ACCM-2 agarose was observed if organisms were plated after 4 days of recovery in ACCM-2. Almost no colonies were observed prior to this point. Based on colony counts, an estimated 8×10^4 pJB-CAT and 4×10^4 pJB-KAN transformants were present in ACCM-2 before plating. Assuming that the 1×10^8 viable *C. burnetii* transformants remaining after electroporation underwent eight divisions during recovery in ACCM-2, we estimate a transformation frequency of approximately 5×10^{-5} . Thus, genetic transformants can be recovered for analysis in as little as 16 days using axenic culture: 4 days of transformant recovery

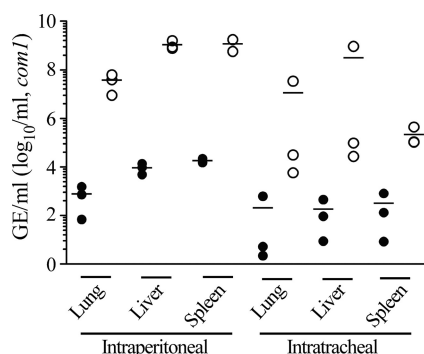


FIG. 5. ACCM-2 supports culture of *C. burnetii* from infected animal tissue. ACCM-2 was inoculated with a 1:100 dilution of total lung, liver, or spleen homogenates derived from NMII-infected SCID mice infected by intraperitoneal or intratracheal routes as described in Materials and Methods. Closed symbols indicate the starting numbers of GE/ml. Open symbols indicate the numbers of GE/ml after a 7-day incubation. Depicted data are the means of three ACCM-2 cultures individually started with tissue homogenates from three infected mice.

in ACCM-2, 6 days of colony development in ACCM-2 agarose, and 6 days of colony expansion in ACCM-2.

ACCM-2 supports growth of *C. burnetii* isolated from animal tissue. Cell culture-based methods of isolating virulent *C. burnetii* from clinical samples have variable success rates (13, 27). Therefore, we tested whether ACCM-2 can be used to isolate *C. burnetii* from animal tissue. As a model, NMII-infected SCID mice were employed (1, 23, 25). Mice were infected intratracheally or intraperitoneally, and lungs, liver, and spleen were harvested at 7 days postinfection. Five ml of ACCM-2 was inoculated with a 1:100 dilution of tissue homogenate, and growth over 7 days was measured by Q-PCR (Fig. 5). Based on the starting number of GE/ml, NMII burdens were highest in tissues harvested from mice infected intraperitoneally. NMII was recovered by axenic cultivation from all tissues, with GE increases of several logs observed for all inocula. The method was highly sensitive, with several cultures successfully started with fewer than 100 total *C. burnetii* GE. In fact, one 5-ml culture was started with 10 GE contained in diluted lung homogenate from a mouse infected intraperitoneally (Fig. 5).

DISCUSSION

In the current study, we describe improved ACCM-based culture methods and their use in a fully axenic system for *C. burnetii* genetic transformation and isolation of the pathogen from tissue. Culturing of *C. burnetii* in gently shaken ACCM-2, containing M β -CD instead of FBS, results in an approximately 1.5 log increase in bacterial yield compared to that obtained when culturing in ACCM. Pathogen generation time in ACCM-2 is reduced to 4.7 h, almost half of that observed in ACCM. In addition, average colony size is increased by about 5-fold to a diameter of approximately 0.5 mm, greatly facilitating the isolation and characterization of clonal populations of *C. burnetii*.

The use of ACCM-2 in *C. burnetii* genetic transformation has several advantages over the previously described Vero cell system of transformant propagation (3). First, the time re-

quired to isolate clonal populations of *C. burnetii* transformants is reduced from 2 to 3 months to as little as 16 days. Second, axenic culture allows recovery of some *C. burnetii* transformants incapable of intracellular growth. Third, transformation frequency can be estimated.

The substitution of M β -CD for FBS in ACCM-2 improves *C. burnetii* growth. Cyclodextrins can also replace FBS in *Helicobacter pylori* culture medium (19) and similarly improves growth of *Bordetella pertussis* (17). Improved growth may result from M β -CD sequestration of inhibitory metabolites, such as certain fatty acids (11).

C. burnetii colony size increases as the agarose percentage decreases (A. Omsland and R. A. Heinzen, unpublished observations). By mixing the bacterial inoculum with a low-density (0.1%) ACCM-2 agarose top medium, colonies develop to approximately 0.5 mm in diameter. By applying the overlay agarose containing *C. burnetii* to a higher-density (0.25%) agarose base, a stable matrix is achieved that allows handling of plates with minimal disruption of the overlay medium. We are uncertain why *C. burnetii* colonies are so small, but potential explanations include the presence of a toxic component in the agarose or the release by *C. burnetii* of a metabolite that changes the microenvironment, such as a basic compound that neutralizes local pH, thereby inhibiting *C. burnetii* metabolism (14). Alternatively, the observation that colonies are primarily comprised of SCVs, a stationary-phase cell form, suggests that the agarose immediately adjacent to colonies has been depleted of nutrients. Despite the colonies' small size, *C. burnetii* displays a nearly one-genome-per-colony plating efficiency in ACCM-2 agarose, indicating that a conventional CFU assay can be used to accurately assess *C. burnetii* viability.

Approximately 6 GE of avirulent *C. burnetii* in phase II is required to produce one infectious focus-forming unit (FFU) in Vero cells (8). In contrast, roughly 300 GE of virulent *C. burnetii* in phase I is required to produce a single FFU (R. A. Heinzen, unpublished data). This behavior is attributed to the hydrophilic nature of phase I *C. burnetii*, conferred by O-antigen sugars, which nonspecifically inhibit host cell plasma membrane interactions relative to hydrophobic phase II organisms, with a truncated lipopolysaccharide (LPS) (28). The low infectivity of virulent phase I *C. burnetii* for cell culture likely lowers the success rate of primary isolation using tissue culture systems. Here, we demonstrate that mouse tissue samples containing as little as 10 NMII GE can initiate cultures in ACCM-2. Thus, axenic culture in ACCM-2 should provide a sensitive method for isolating virulent *C. burnetii* in clinical samples. Indeed, recent large Q fever outbreaks in Europe (12, 26) reinforce the need for such an enhanced surveillance and diagnostic tool.

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